PHYLOGENETIC RESOLUTION POTENTIAL OF 18S AND 28S rRNA GENES WITHIN THE LITHISTID ASTROPHORIDA

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Kelly-Borges et al. (1991) and Kelly-Borges & Pomponi (1994) utilised partial 18S rRNA gene sequences to resolve relationships within hadromerid and lithistid sponges (Porifera: Demospongiae). While their results clarified several specific systematic problems, their conclusions were hampered by low levels of sequence variation. This study sought primarily to evaluate the resolution potential between regions of the 18S rRNA gene used in previous studies on sponges, and 28S rRNA genes used in more recent work. Six lithistid sponge taxa were chosen to represent a gradient of taxonomic relationships, ranging through genus, family, order and class. Approximately 1,300bp of the 18S rRNA gene and a 700bp region at the 5' end of the 28S rRNA gene were compared with the data of Kelly-Borges & Pomponi (1994). We found that the 700bp region of the 28S rRNA gene presented the greatest potential for resolution of this group of Porifera at the genus and family level, and that the resultant molecular phylogeny was congruent with morphological hypotheses for the group.
❒ *Porifera, molecular phylogeny, evolution, Lithistida, Theonella, Discodermia, Corallistes.*

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For the past twenty years, organismal phylogenies have been inferred from the primary sequence of a portion of their genomes. The small subunit ribosomal RNA (SSU rRNA) or 18SrRNA gene has dominated the field as molecule of choice. The analysis of this molecule has been singularly instrumental in elueidating the phylogenetic relationships and natural history of almost all known prokaryotic species where attempts using other methods have failed (see Woese, 1987). Following the success of microbiologists adopting this approach, the systematics of eukaryotic taxa has been addressed by sequencing the 18S rRNA gene (Sogin et al., 1986). This gene is probably still the most frequently used for this purpose.

It is often desirable to use more than a single gene region for the reconstruction of a phylogeny, to supply additional, potentially eorroborative phylogenetie hypotheses. For example, the 5' region of the 28S rRNA gene has also been used with great effeetiveness (Baroin et al,. 1988; Chombard et al., 1998), as have Elongation Factor genes (Iwabe et al., 1989; Rivera & Lake, 1992), ATPases (Iwabe et al., 1989) and DNA-dependent RNA polymerases (Puhler et al. 1989), among others. There are a number of additional prerequisites for choosing a gene for the purposes of reconstructing a phylogeny. It is desirable that a eonstaney of funetion (funetional orthology) is maintained throughout the evolution of the taxa of interest. Problems assoeiated with long branches may be observed on trees where some genes have experieneed a relaxation in selective pressures. The possibility of mistakenly isolating a paralogous homologue must be minimal. A suitable genc must show signs of having enough variability to discriminate between taxa at the desired taxonomic lcvel. It must also be conserved enough to permit robust alignments and comparisons across the deepest divisions.

Sponge phylogenies, in particular, have given rise to a number of eontentious arguments, most of which result from a laek of suitably variable morphologieal characters which distinguish sponges at the species level and higher (Van Soest, 1987; Hadju et al., 1993; Kelly-Borges & Bergquist, 1997; Sandford & Kelly-Borges, 1997). The primary diagnostic morhological eharacters that differentiate sponge genera and species are spicule morphology and their arrangement within the sponge body. Characters often influeneed by environmental factors, such

as texture, surface features and colouration are less reliable as they are frequently plastic.

Although the construction of poriferan phylogenetic hypotheses using molecular sequence data is still at a preliminary stage, with very few studies completed, it is likely that many future sponge systematic projects will incorporate a molecular moiety. For this reason, it is necessary to establish the taxonomic levels at which certain gene regions will be appropriate. For example, some stretches of DNA might be informative about phylogenetic relationships at the genus and species level, but they may be unsuitable for studies of ordinal relationships and so on. All studies so far have used different genes and gene regions for phylogenetic purposes (Kelly-Borges et al., 1991, 1994; West & Powers, 1993), preventing any useful links between these data towards the construction of larger phylogenies. The advantage that may be gained in future by using the same gene region in all studies is therefore obvious.

The primary goals of our study were to evaluate the resolution potential between various regions of sponge 18S and 28S rRNA genes, in order to determine which genes would efficiently resolve phylogenies at several taxonomic levels. Because of past difficulties in resolution using 18S rRNA (Kelly-Borges et al, 1991; Kelly-Borges & Pomponi, 1994), we took a positive approach in our more recent research to determine which gene would successfully provide resolution within a group of lithistid sponges, and thus, potentially within other taxonomic groups.

In this study we evaluate the relative utility of four alignments with different gene origin, sequence length, and method of analysis. To do this, we extended the 18S rRNA gene data of Kelly-Borges & Pomponi (1994) for six species up to approximately 1,300bp, and in addition, using the same taxa, we have sequenced approximately 700bp of the 5' end of the 28S rRNA gene. Taxa were selected to encompass a range of taxonomic levels including genus, family, order, and class. The criteria by which the sequence data sets were evaluated for their potential utility included counting the number of variable sites and the number of parsimony-informative sites, using maximum likelihood in order to estimate the proportion of constant sites that might be invariable, and also conducting an objective analysis of the resulting tree topologies. The resulting topologies were compared with a hypothetical reconstruction based upon morphological characters.

For the purpose of this exercise four lithistid sponges (Class Demospongiae) were selected from a much larger study on sponge phylogeny, and two hexactinellid sponges (Class Hexactinellida) were chosen as an outgroup. Lithistid sponges represent relict forms of an ancestral fauna from which, it is thought, most demosponges have evolved. These sponges are characterised by the possession of a rigid siliceous skeleton made up of irregular branching desma spicules, the ends of which interlock (zygose) with neighbouring spicules (see Kelly-Borges & Pomponi, 1994, Figs 1,2). In some cases there are additional spicules present, providing clues as to their affinities with non desma-bearing sponges, and the polyphyly of at least some of these genera has been recently confirmed by Kelly-Borges & Pomponi (1994). Although very difficult to differentiate morphologically, the most reliable diagnostic characters that can be used are the morphology, ornamentation, and pattern of zygosis of the desma spicules, and the morphology of the additional spicules if they are present.

MATERIALS AND METHODS

TAXA SELECTION. Sponge species were selected from a broader study of sponge phylogeny, specifically for the purpose of examining capability of taxonomic resolution of poriferan sequence data (Table 1). All sponges were collected using Harbor Branch Oceanographic Institution's 'Johnson-Sea-Link II' manned submersible, except *Theonella* spp. which were collected using SCUBA. Samples were identified through histological examination of skeletal structures, the procedures for which are detailed in Kelly-Borges et al. (1994). Voucher specimens have been deposited in the collections of either The Natural History Museum, London (BMNH), or the Harbor Branch Oceanographic Museum, Florida (HBOM). Registration numbers are given in Table 1. Hexactinellid sponges *Margaritella coeloptychioides* and *Sympagella nux* (Table 1) were selected to provide outgroup sequences for the molecular phylogeny reconstruction.

MORPHOLOGICAL PHYLOGENY RECONSTRUCTION. A phylogenetic analysis of morphological characters (Table 2) was carried out to examine relationships within and between the theonellid and corallistid taxa for the purpose of comparison with trees gained from reconstruction of molecular data. The analysis used the

TABLE 1. Collection data and taxonomic position for sponge taxa sequenced in this study.

Taxon	Museum Registration	Locality
Class Hexactinellida		
Subclass Hexasterophora		
Order Hexactinosida		
Family Euretidae		
Margaritella coeloptychioides Schmidt, 1870	HBOM 003:00925	Turks and Caicos
Order Lyssacinosida		
Family Caulophacidae		
Sympagella nux Schmidt, 1870	HBOM 003:00929	Turks and Caicos
Class Demospongiae		
Subclass Tetractinomorpha		
Order Astrophorida		
Family Theonellidae		
Theonella sp. 1	BMNH1998.3.4.1	Belau, Micronesia
Theonella sp. 2	BMNH1998.3.4.2	Belau, Micronesia
Discodermia sp.	BMNH1998.3.4.3	Bahamas, Caribbean
Family Corallistidae		
Corallistes typus	Schmidt, 1870	BMNH1998.3.4.4

TABLE 2. Morphological characters (A) and their character-states (B) for taxa in this study (see Kelly-Borges & Pomponi, 1994, for an explanation of characters). Characters indicated as * are absent from the outgroup in that they do not possess desmas.

A. Character number	Character	Character state
1	Desmas.	a, present; b, absent
2	Desma development.	a, tetraclonal; b, dicranoclonal; c, absent*
3	Zygosis architecture.	a, articulated at ends of zygomes (Fig. 2A,C); b, articulated along zygomes (Fig. 2E); c, absent*
4	Monactinal megascleres.	a, oxea; b, tylostrongyles (Fig. 2C); c, whispy oxeotes (Fig. 1A)
5	Triaene megascleres.	a, short-shafted discotriaenes (Figs 1C, 2B); b, short-shafted phyllotriaenes (Fig. 2D); c, long-shafted ornamented dicho- and trichotriaenes (Fig. 1A,B); d, long-shafted ortho- and dichotriaenes
6	Euasters.	a, present; b, absent
7	Streptasters (Fig. 2F).	a, present; b, absent
8	Small acanthose microrhabds (Fig. 2B).	a, present; b, absent
9	Large acanthose microrhabds (Fig. 2B).	a, present; b, absent

Branch and Bound search option of PAUP 3.1.1, and data were unordered and unweighted. In order to obtain a directed analysis, members of two non-lithistid astrophorid families, *Geodia* (Family Geodiidae) and *Stelletta* (Family Ancorinidae) were chosen as outgroups. The major skeletal characters that separate these lithistids from their outgroups are the possession of desmas, unique ornamented dichotriaenes, and certain types of microscleres (see Kelly-Borges & Pomponi, 1994).

MOLECULAR EXPERIMENTAL PROCEDURES. Sample collection, preservation and DNA extraction have been previously described (Kelly-Borges & Pomponi, 1994). PCR primers and sequencing oligonucleotides are listed in Table 3 for both genes. PCR reactions were carried out in a 50µl reaction volume which contained a one-tenth volume of 10x PCR buffer (500mM KCl, 100mM Tris-HCl, 1% Triton X-100), dNTPs to a final concentration of 200µM, primers at a concentration of 200µM and 2.5mM MgCl. The PCR protocol began with an initial denaturation at 94°C for 5mins, followed by 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min and extension at 72°C for 1min. This PCR regime was used for both genes. Following cycling, the success of the amplification was determined by electrophoresing on an ethidium bromide-stained agarose gel and visualised by short-wave UV illumination. For each taxon, a total of 10 PCR reactions were carried out and the products were pooled. This was an effort to reduce the potential for an amplification-induced error in the sequences. The pooled amplification products were electophoresed on a single agarose gel and the band was excised using a clean scalpel blade. The DNA

B. Taxon	1	2	3	4	5	6	7	8	9
Discodermia	a	a	a	a	a	b	b	a	a
Theonella sp. 1	a	a	a	b	b	b	b	a	b
Theonella sp. 2	a	a	a	b	b	b	b	a	b
Corallistes sp.	a	b	b	c	c	b	a	b	b
Geodia (outgroup)	b	c	c	a	d	a	b	b	b
Stelletta (outgroup)	b	c	c	a	d	a	b	b	b

fragment was extracted from the agarose using the Quiaex II (Quaigen Ltd, U.K.) PCR purification kit. DNA sequencing reactions were carried out using the Amplitaq FS sequencing kit (Applied BioSystems, Inc.). The sequencing protocol for both genes was carried out as per the manufacturer's instructions. Sequencing was carried out on an Applied Biosystems 373 automated sequencing apparatus and the data was analysed using the Sequence Navigator software (Applied Biosystems Inc.). We estimate that 98% of gene regions were covered by more than one sequencing read, with approximately 25% being covered by three or more sequencing overlaps. The sequences have been deposited in the EMBL sequence repository under the accession numbers AJ224646-AJ224651 (demosponge 18S rRNA), AJ224123-AJ224124 (hexactinellid 18S rRNA), AJ005911-AJ005918 (28S rRNA).

MOLECULAR PHYLOGENY RECONSTRUCTION. Nucleotide positions whose identities were not possible to establish unambiguously, were coded according to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature. The sequences were aligned using the Genetic Data Environment (Smith, 1993). In the majority of cases, the positional orthology of the nucleotides was relatively easy to establish. A conservative approach to the alignment was taken, with those positions whose homology was not possible to establish with absolute certainty, being excluded from subsequent analyses. Attempts in the most difficult cases, to relate the sequences to each other, on the basis of RNA secondary structure proved recalcitrant. In the absence of conclusive grounds for establishing homology, the sites in question were excluded. Phylogenetic hypothesis construction and sequence statistics were evaluated using PAUP*4.0d54 (Swofford, 1993). Transition-transversion ratios were calculated from each dataset by first constructing a neighbor-joining tree from LogDet distances (Lockhart et al., 1994). This tree was considered a working hypothesis of relationships. The estimate of transition-transversion ratio might be influenced to some extent by tree topology, but this influence was not thought to be significant. Using maximum likelihood criteria, the transition-transversion ratio was chosen that yielded the highest likelihood value. The gamma shape parameter for each dataset was calculated using the optimised transition-transversion ratio, again using maximum likelihood as the optimisation criterion. The gamma shape parameter that yielded the highest likelihood was chosen.

RESULTS

MORPHOLOGICAL PHYLOGENY RECONSTRUCTION. A single minimum length tree was obtained of length 14 and a very high consistency index (CI) of 1.0. The phylogenetic tree hypothesises that species of *Theonella* are more recently derived than *Discodermia*, and that these two genera form a clade more recently derived than *Corallistes*. This topology is identical to one of the reconstructions derived from sequence data (Fig. 3A), and supports the current classification that recognises the differentiation of *Discodermia* and *Theonella* from *Corallistes* in the Family Theonellidae and Family Corallistidae, respectively (see Kelly-Borges & Pomponi, 1994).

Morphological characters 2,3,7, 8 and 9 (Table 2) differentiate *Discodermia* and *Theonella* from *Corallistes*, and the states of characters 4 and 5 differentiate *Theonella* from *Discodermia*, and both from *Corallistes*. In *Discodermia* and *Theonella* the desmas are tetracrepid (character 2a) with four clones (zygomes) which clasp (zygose) at their very ends (character 3a; Fig. 2A,C). *Corallistes*, on the other hand, has dicranoclonal desmas (character 2b) in which zygosis spreads along the zygomes (character 3b; Figs 1A, 2E).

The desma skeleton of these genera is supplemented with monaxonal megascleres - oxea in *Discodermia* (character 4a), long and curved with blunt hammer-like ends (tylostrongyles) in *Theonella* (character 4b; Fig. 2C), and whispy roughened oxea-like spicules in *Corallistes* (character 4c, Fig. 1A). Triaenose megascleres are found at the surface of the sponge with their head rays parallel with the surface and the rhabd perpendicular to the surface (Fig. 1A,C). These are discotriaenes in *Discodermia* (character 5a; Fig. 1C, 2B), phyllotriaenes in *Theonella* (character 5b; Fig. 2D), and ornamented (character 5c; Fig. 1A,B) or plain (Fig. 2F) dichotriaenes in *Corallistes*. Microscleres are scattered throughout the sponge body and often form a thick surface crust. In *Discodermia* there are two size categories of roughened microrhabds (characters 8a, 9a; Fig. 2B), whereas in *Theonella* there is only one (character 8a; Fig. 2C). *Corallistes* lacks microrhabds but possess streptasters (Fig. 2F).

TABLE 3. Oligonucleotide names and their corresponding sequences. The first and second oligonucleotides are designed to amplify a large portion of the 18S rRNA gene, whilst the third and fourth oligonucleotide sequences are designed to amplify an approximately 700bp stretch of the 28S rRNA gene.

Oligonucleotide name	Sequence
18Sf20	TGG TAC GGT AGT GGC CTA CCA TGG
18Sr21	ACG GGC GGT GTG TAC AAA GGG CAG
RD3A	GAC CCG TCT TGA AAC ACG A
RD5B2	ACA CAC TCC TTA GCG GA

MOLECULAR PHYLOGENY RECONSTRUCTION. In total, six taxa had a portion of their 18S genes sequenced. The final alignment was in excess of 1,300bp in length. The positions whose alignment could not be determined unambiguously were removed. When all taxa were considered, the final alignment was 1,135 positions in length, whereas with the exclusion of the hexactinellid sequences, the number of alignable positions increased to 1,269. For the 28S rRNA gene dataset, almost 700bp were sequenced for each taxon. The final alignment lengths were 505 positions for the eight taxon dataset and 583 positions for the ingroup taxa alone.

A total of eight alignments were analysed for their information content. Statistics that were evaluated included the number and percentage of variable sites, the number and percentage of parsimony-informative sites, the estimated gamma shape parameter for rate variation across sites and the transition-transversion ratio. The results of these analyses are given in Table 4. The top half of the table refers to the alignments that were used when the two hexactinellid outgroup sequences were included, whereas the bottom half of the table refers to the ingroup only (in this instance lithistid demosponges only).

In most cases, the exclusion of the hexactinellid outgroup sequences facilitated the use of a longer gene region. This was due to the difficulty of aligning some hexactinellid regions with their equivalent location in the demosponge genes. On the other hand, removal of the hexactinellid sequences had the effect of reducing the numbers of variable and informative sites, with a consequent increase in the number of constant sites. The combined dataset always contained the highest number of variable and informative sites as would be expected, but in neither instance did it contain the greatest percentage. One of the striking features of both kinds of analysis is the performance of the 28S rRNA gene dataset. This region had the highest

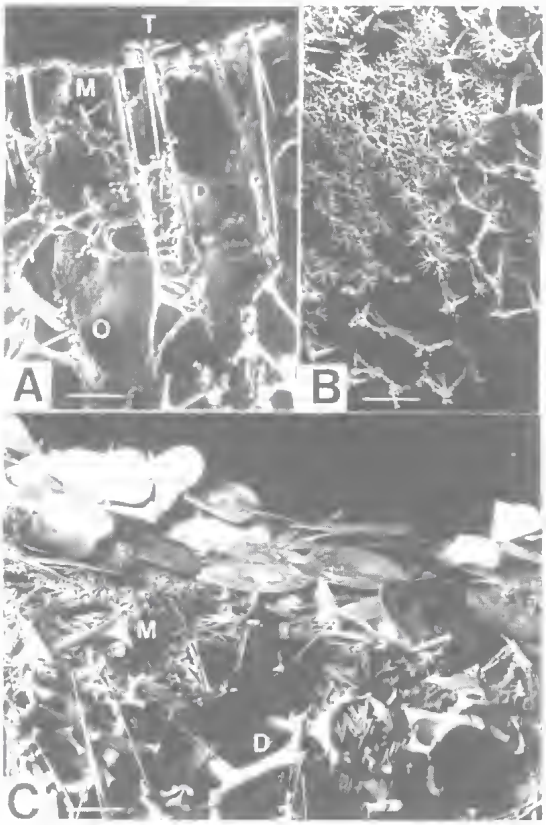

FIG. 1. Skeletal architecture of lithistid demosponges. Transverse sections have been taken through the sponge surface, cellular material removed using hydrogen peroxide, and viewed by SEM. A, *Corallistes nolitangere*. Transverse section through surface of sponge showing dicranoclonal desma reticulation (D). Long-shafted ornamented dichotriaenes (T) emerge from the desma reticulation with the shaft perpendicular to the surface and the cladomes (head) parallel with the surface. Oxeote spicules (O) resembling fine hairs can be seen in residue cellular material. Streptaster microscleres pack the surface (M). Scale=238μm. B, *Corallistes nolitangere*. View of the sponge surface showing the ornamented heads of the dichotriaenes, with the desma reticulation visible beneath. Scale=400μm. C, *Discodermia* sp. Transverse section through surface of sponge showing tetracrepid desma reticulation (D) and above this a dense crust of two sizes of acanthose microrhabd microscleres (M). Short-shafted discotriaenes line the surface with discs overlapping. Scale=111μm.

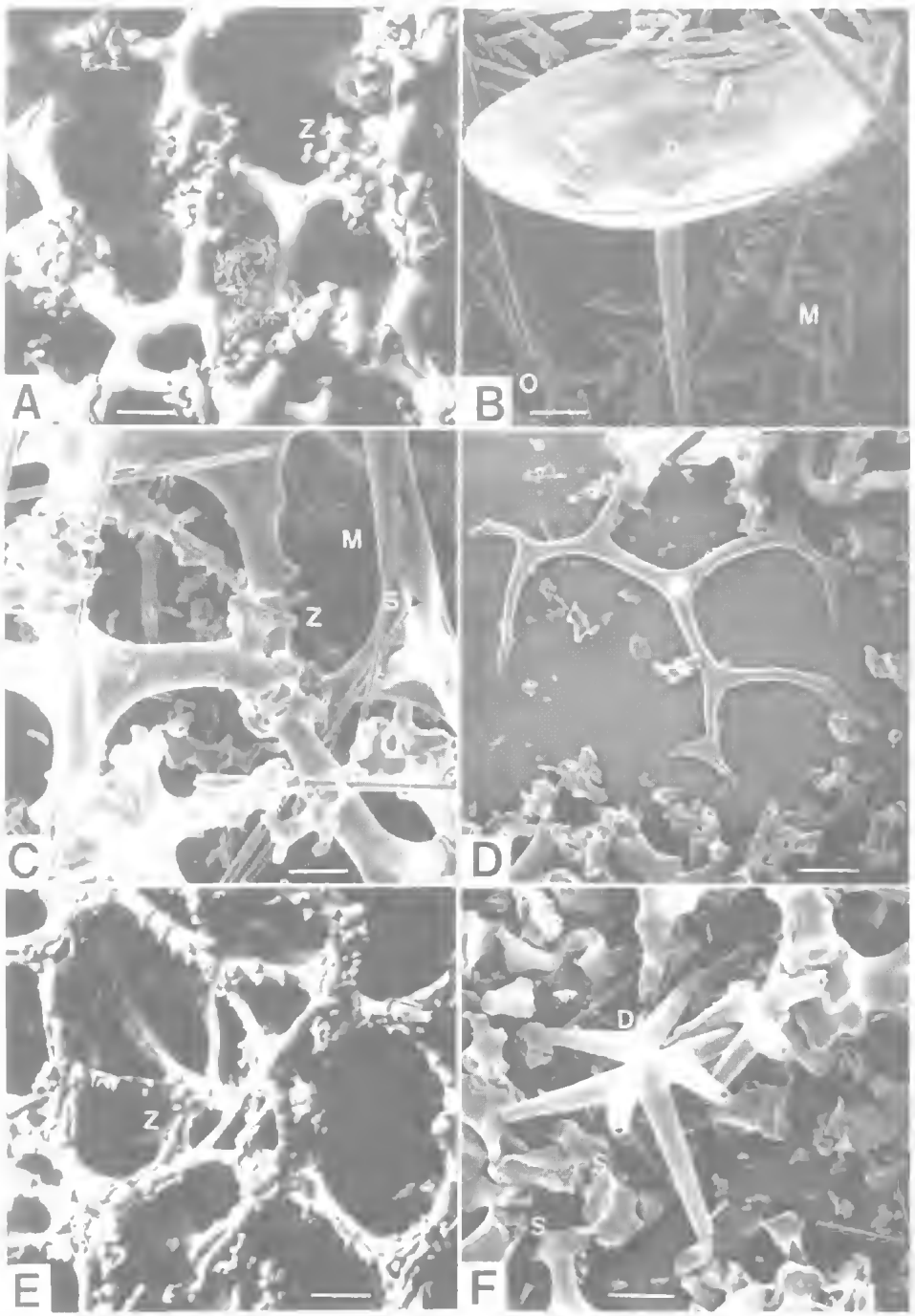

FIG. 2. Desma, triaene and microsclere morphology in *Discodermia*, *Theonella* and *Corallistes*. A-B, *Discodermia* sp. A, Tetracrepid desmas with tuberculate zygoses (Z) at the ends of the zygomes. Scale=125μm; B, Short-shafted discotriaene (D), large and small acanthose microrhabd microscleres (M), and large regular oxea (O). Scale=20μm. C-D, *Thenonella* sp. C, Tetracrepid desmas with zygoses (Z) at the ends of the zygomes. Acanthose microrhabd microscleres are of one size (M) and strongyles have tylote ends (S). Scale=30μm. D, Short-shafted phyllotriaene. Scale=50μm. E-F, *Corallistes nolitangere*. E, Dicranoclonal desmas with zygoses (Z) along the zygomes. Scale=100μm; F, Long-shafted dichotriaene (D), streptaster microscleres (S). Scale=50μm.

TABLE 4. Results from analyses of eight alignments. The top half of the table refers to the alignments that were used when the two hexactinellid outgroup sequences were included, whereas the bottom half of the table refers to the ingroup only (i.e. lithistid demosponges only), where *Sympagella nux* and *Margaritella coeloptychioides* were excluded. The first column contains the gene region that was used for each particular analysis. The words 'all taxa' in parentheses indicates that all six taxa were used in that particular analysis. 18S (short) refers to the data of Kelly-Borges & Pomponi (1994).

Gene region	Length	Variable	Informative	Gamma	Ti/Tv
18S (long) (all taxa)	1138	446 (39%)	163 (14%)	0.31	0.98
18S (short) (all taxa)	473	137 (29%)	75 (16%)	0.24	1.20
28S (all taxa)	505	142 (28%)	84 (17%)	0.24	1.79
Total (all taxa)	1643	588 (36%)	247 (15%)	0.29	1.18
18S (long) (ingroup)	1269	214 (17%)	37 (3%)	0.54	1.16
18S (short) (ingroup)	473	72 (15%)	8 (2%)	0.79	1.46
28S (ingroup)	583	169 (29%)	40 (7%)	0.40	1.26
Total (ingroup)	1852	383 (21%)	77 (4%)	0.34	1.19

percentage of parsimony-informative sites in both datasets and when outgroup taxa were removed, it also had the highest percentage of variable sites.

The gamma shape parameter, which is an estimate of the rate variation across the sites in the alignment, ranged from 0.24-0.31 for alignments that included all taxa, and from 0.34-0.79 for ingroup alignments. The increased estimate of rate variation across sites (lower gamma value) in the eight taxon datasets indicates that the addition of more sequences caused more variation at sites that were already variable, whilst conserved sites remained so even with the addition of more distant taxa.

Of the twelve possible substitution types, there are twice as many possible transversion mutations as transition mutations. For these datasets, the maximum likelihood transition-transversion ratio is slightly above 1.0 in most cases (except for the 18S rRNA dataset with the eight taxon alignment). This is indicative of a bias towards transition substitutions. At very extreme genetic distances, the transition-transversion ratio will converge to 0.5. This is not apparent in any of the datasets in these analyses.

The short 18S alignments generally produced trees with low amounts of resolution. Frequently none of the hypothesised phylogenies in Figure 3 were seen in the resulting bootstrap partition tables. The longer 18S alignments provided a greater amount of resolution, with bootstrap proportions sometimes becoming quite high. The 28S alignments were also quite well resolved and these also yielded high bootstrap values, particularly for the phylogeny that is

Topology		18S (long)		28S		18S (short)		Total	
		All taxa	Ingroup	All taxa	Ingroup	All taxa	Ingroup	All taxa	Ingroup
a Theonella sp1, Theonella sp2, Discodermia, Corallistes	LD	8	42	77	96	47	49	54	90
	ML	8	4	87	86	9	0	59	57
	P	7	0	84	62	0	0	45	13
b Theonella sp1, Theonella sp2, Discodermia, Corallistes	LD	51	43	18	2	0	0.33	42	10
	ML	50	65	13	14	23	49	32	38
	P	83	97	16	38	0	0	55	87
c Theonella sp1, Theonella sp2, Corallistes, Discodermia	LD	31	14	0	0	34	11	1	0
	ML	42	31	0	0	0	0	9	5
	P	10	3	0	0	0	0	0	0

FIG. 3. Phylogenetic reconstructions of the relationships between *Discodermia*, *Theonella* and *Corallistes*. The first column (topology) indicates the topology that is under consideration and the open circle indicates the internal branch whose support is being assessed. The second column indicates the type of analysis that was undertaken: LD - LogDet; ML - Maximum Likelihood; P - Parsimony. The results are in four consecutive blocks according to the gene region that was used in the analysis. Within each block, the left side indicates the bootstrap proportions for the alignment that included all taxa and the right side indicates the results when only the ingroup sequences were used.

favoured by the morphological data (Fig. 3A). The combined sequence dataset alignments also displayed a reasonable amount of signal.

DISCUSSION

The analysis of sequence statistics showed that the 28S rRNA gene region provides the greatest amount of information per unit sequence length (Table 4). Despite the fact that the 28S alignment was hampered by the necessity of removing a large hypervariable portion, it still contained a high number and percentage of variable and parsimony-informative sites. The 18S gene behaved in a less efficient way. The combined dataset always contained the largest number of variable and parsimony-informative sites, but it did not contain the highest percentage of these sites in any of the analyses.

It is curious to note the behaviour of the estimated shape of the gamma parameter, a. The gamma shape parameter is an estimation of rate variation across sites. Lower gamma parameters indicate a more severe amount of rate variation whilst higher numbers indicate that the evolutionary rate is more equivalent at all sites. There was an obvious difference in values between the taxon-inclusion sets. When all taxa were considered, the gamma shape parameter was always lower than when only ingroup taxa were analysed. The addition of more taxa is simply increasing the amount of variability at sites that are already free to vary. Conserved sites remain unchanged with the addition of more taxa. Although the addition of more taxa has the effect of increasing the percentage of variable and parsimony-informative sites, the gamma shape of rate variation across sites is more marked.

Not all of the three phylogenetic trees were congruent with the morphological phylogeny. The shorter of the two 18S rRNA alignments was unable to resolve the relationships of the four taxa of interest with any degree of confidence. Indeed during some bootstrap replicates, some other topologies, not considered in Figure 3 were found. The main reason for this was a complete lack of variability in the dataset.

The longer 18S gene region was slightly more decisive about branching order. The topology that received strong support using this region was a pairing of *Discodermia* and *Corallistes* to the exclusion of the other taxa. However, this high level of support was only achieved using parsimony tree reconstruction. Given the general lack of confidence using the other methods, and the inability of parsimony to compensate for superimposed substitutions, it is possible that these findings are a result of long branch artifacts. This clade is rooted by a particularly long branch leading to the other demosponge taxa. The topology that was observed the least number of times during bootstrapping was the pairing of *Discodermia* with *Theonella*, and this was irrespective of type of analysis or taxon-inclusion set.

The results for the 28S gene sequences were considerably different to those seen in the 18S analyses. For this gene, the placement of *Corallistes* as the sister taxon to *Theonella* was never seen in any analysis (Fig. 3C) Of the two remaining alternative topologies, the placement of *Discodermia* as sister taxon to *Theonella* (Fig. 3A) received considerably more support than the placement of *Discodermia* with *Corallistes* (Fig 3B). Reasonably high bootstrap support was seen for *Discodermia* and *Theonella* as sister-groups using all methods of analyses irrespective of whether the outgroup taxa were used or not.

The alignment combining 18S and 28S rDNA data also yielded ambiguous results. The topology that suggests a sister taxon relationship between *Corallistes* and *Theonella* is very poorly supported (Fig. 3C). The other two topologies are more strongly supported, but neither was supported with any degree of confidence and the differences in the levels of support do not justify acceptance of one over the other. It is likely that the ambiguous nature of the results from the 18S rRNA gene have a detrimental effect on the combined alignment. During bootstrapping, a character can be selected from either gene region. Given that the 18S gene region is approximately 150% larger than the 28S rRNA gene region, it will probably contribute more to each replicate on the whole. The result of this seems to be the carry-over of the ambiguous results from the separate analysis that used only the 18S rRNA gene.

The topology that was most strongly supported using the 28S gene is consistent with the hypotheses of relationships deduced from morphological characters; *Discodermia* and *Theonella* are more closely related to each other than they are to *Corallistes*, and they are the more derived taxa. The 28S rRNA gene generally has a higher proportion of variable and parsimony-informative sites and can provide the best possibility of resolving poriferan phylogenetic relationships, at least at the sub-ordinal level.

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